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## An HLA-C-Restricted CD8+ Cytotoxic T-Lymphocyte Clone Recognizes a Highly Conserved Epitope on Human Immunodeficiency Virus Type 1 *gag*

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A unique epitope on the *gag* protein of human immunodeficiency virus type 1 (HIV-1), located at amino acid 145 to 150, has been mapped by using a CD8+ cytotoxic T-lymphocyte (CTL) clone. This epitope is highly conserved among 18 HIV-1 strains. The HIV-1 *gag*-specific human leukocyte antigen (HLA) class I-restricted CD8+ CTL clone was generated from fresh peripheral blood mononuclear cells of an HIV-seropositive donor by stimulation with  $\gamma$ -irradiated allogeneic peripheral blood mononuclear cells in the presence of an anti-CD3 monoclonal antibody and recombinant interleukin-2. This *gag*-specific CTL clone killed autologous target cells infected with a recombinant vaccinia virus containing the *gag* gene of HIV-1 and target cells pulsed with an authentic p24<sup>gag</sup> construct expressed in *Escherichia coli*. Fine specificity was determined by using a panel of overlapping 30-amino-acid-long synthetic peptides and subsequently using smaller peptides to precisely map the CTL domain on p24. The epitope is on a highly conserved region, and it overlaps with a major B-cell epitope of *gag*. This CD8+ T-cell epitope is restricted by HLA-Cw3, which has not been previously identified as a restricting element for human CTL responses.

The development of vaccines against AIDS has focused primarily on the viral envelope gene product and has been complicated by the emergence of neutralization escape mutants (10, 12, 14) as well as sequence variation in the envelope glycoprotein (40, 44). Previous candidate vaccines that induced human immunodeficiency virus (HIV)-specific neutralizing antibodies in chimpanzees did not protect these animals from infection after challenge with live virus (14). A recent report demonstrating protection of these animals against homologous virus challenge for more than 6 months after vaccination with recombinant glycoprotein gp120 leaves questions about the length of this protective immunity (1). For a vaccine to be effective in humans, it should provide protection for a long period after vaccination. It should also elicit specific cytotoxic T-lymphocyte (CTL) effectors that are able to eliminate cells which are infected by virus that has escaped neutralization, either because of antigenic drift or because of concentrations of antibody inadequate to completely prevent infection. Efforts have thus been focused on studying CTL responses against different HIV proteins; however, only a few epitopes recognized by human CTL clones have been described.

A DR4-restricted gp120 epitope (amino acids [aa] 410 to 429) recognized by CD4+ clones has been identified, but this region is not conserved in other HIV-1 strains (40). Several

epitopes defined by reverse transcriptase-specific CTL clones that were restricted by human leukocyte antigens (HLA) A and B have been reported (50). Koenig et al. (16) demonstrated an epitope of 10 amino acids (aa 73 to 82) within *nef* recognized in association with HLA-A3 by a CD8+ CTL clone. The only available information concerning CTL epitopes on *gag* have been an HLA-B27-restricted epitope on p24<sup>gag</sup>, analyzed by using bulk stimulated peripheral blood mononuclear cells (PBMC) as effector cells (26) and HLA-A2-restricted epitopes that were described in bulk cultures (5). In contrast to the envelope glycoprotein, there is less heterogeneity exhibited by the internal viral proteins of sequenced HIV-1 isolates, which makes characterization of T-cell responses of these proteins a priority in view of AIDS vaccine design. In this report, we describe the first CTL response restricted by HLA-Cw3. This HLA antigen is quite common, and this epitope on p24 is highly conserved among HIV-1 strains.

### MATERIALS AND METHODS

**Recombinant vaccinia viruses and proteins.** Vaccinia virus (VAC) and three previously described recombinant vaccinia viruses (VAC/*gag*, VAC/*pol*, and VAC/gp160) expressing the *gag* (48), reverse transcriptase (50), and envelope (4) genes of HIV-1 (IIIB strain), respectively, were used to infect target cells for CTL assays.

Recombinant HIV-1 (IIIB strain) p24<sup>gag</sup>, expressed (6)

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and purified (13a) from *Escherichia coli*, was also used for the preparation of target cells.

**Preparation of synthetic peptides.** Synthetic peptides of HIV-1 p24<sup>gag</sup> were prepared and purified as described previously (11, 29) on the basis of the HIV-1 (III B strain) sequence (34). The initial peptides tested overlapped with adjacent peptides by 10 or 20 amino acids, and subsequently shorter peptides were prepared and analyzed. The numbering of residues is based on the compilation of Myers et al. (24).

**Preparation of target cells.** B-lymphoblastoid cell lines (B-LCL) were established by Epstein-Barr virus transformation of the PBMC from an HIV-1-infected donor (autologous) and from major histocompatibility complex (MHC) HLA class I- and II-matched or mismatched allogeneic donors. Culture supernatant of B95-8 cells was used as an inoculum of Epstein-Barr virus. These B95-8 cells were provided by T. Sairenji of the University of Massachusetts Medical Center.

The B-LCL were either uninfected (control) or infected with vaccinia viruses at a multiplicity of infection of 20 for 12 to 16 h, labeled with sodium [<sup>51</sup>Cr]chromate for 1.5 h, washed thrice, and resuspended to give the appropriate concentration for use as target cells in cytotoxicity assays.

The B-cell lines were also used as targets after overnight pulsing with 100 µg of purified recombinant p24 protein per ml or pulsing with synthetic peptides at a concentration of 20 to 100 µg/ml for 1 h. These B-cell lines were labeled with <sup>51</sup>Cr, incubated for another hour, and then washed thrice before use.

**Preparation of effector cells.** PBMC were obtained from the blood of an asymptomatic HIV-1 antibody-positive individual (Walter Reed stage II) by Ficoll-Hypaque density gradient centrifugation. Cells were resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), penicillin (100 U/ml), and streptomycin (100 U/ml) and used directly as effector cells in cytotoxicity assays. To obtain T-cell clones, PBMC were seeded at 50 cells per well in 96-well plates with  $2 \times 10^5$  γ-irradiated normal PBMC from HIV-1-seronegative donors in 0.2 ml of RPMI 1640 containing 10% fetal calf serum, recombinant human interleukin-2 (100 U/ml; Cellular Products, Buffalo, N.Y.), and anti-CD3 antibody 12F6 (0.1 µg/ml) as described by Walker et al. (49). Twice weekly, 0.1 ml of medium was removed from each well and replaced with fresh medium as described above. On day 14, when cell proliferation was observed in all of the wells, cells were transferred to 48-well flat-bottom plates (Costar) and further cultured with  $10^6$  γ-irradiated allogeneic feeders in 1 ml of the same medium. These cell lines were restimulated every 2 weeks and subsequently used as effector cells in the assay described below.

**Cytotoxicity assays.** Assays were performed in round-bottom 96-well plates. Various concentrations of effector cells in 0.1 ml of RPMI-10% fetal calf serum were added to 0.1 ml of a <sup>51</sup>Cr-labeled target cell suspension ( $10^4$  cells per ml) in the wells to give the described effector/target (E/T) ratios. After a 6-h incubation at 37°C, the supernatant was harvested from each well and counted in a gamma counter. The assays were performed in triplicate wells, and the percent specific cell lysis was calculated by the formula  $100 \times (\text{mean experimental release} - \text{mean spontaneous release}) / (\text{mean total release} - \text{mean spontaneous release})$ . The results of an assay were excluded if the average spontaneous release was  $\geq 30\%$  or if the viability prior to <sup>51</sup>Cr labeling was  $< 80\%$ .

**HLA typing.** Most of the donor lymphocytes used in these

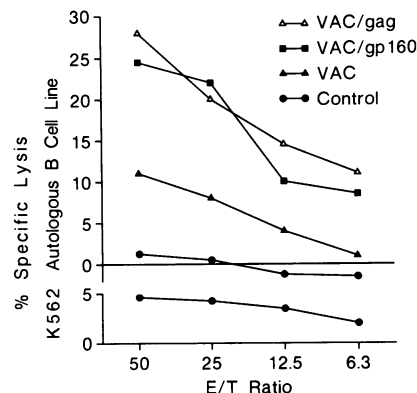


FIG. 1. Cytotoxic activity of fresh PBLs from an HIV-1-seropositive subject, using four different E/T ratios. Autologous <sup>51</sup>Cr-labeled B-LCL were uninfected (control) or virus infected as indicated. The K562 cell line was used as a control to monitor natural killer cell activity.

experiments were typed for HLA-A, -B, -C, and -D antigens on unfractionated PBMC by the Tissue Typing Laboratory at the George Washington University Medical Center. Some donor lymphocytes were tissue typed by using their Epstein-Barr virus-transformed B-cell lines in the Tissue Typing Laboratory at the University of Massachusetts Medical Center.

**Phenotypic analysis.** Cell lines were stained with fluorescein isothiocyanate-conjugated monoclonal antibodies (MAbs) anti-Leu4 (CD3), anti-Leu2 (CD8), anti-Leu3 (CD4), and anti-Leu11a (CD16), purchased from Becton Dickinson and Co. (Mountain View, Calif.). The percentage of antigen-positive cells was analyzed by fluorescence-activated cell sorting (Becton Dickinson model 440 apparatus).

**Antibody blocking of target cell lysis by CTL.** MAbs B7/21.7, S3/4, and OKIa1 recognize DP, DQ, and DR determinants, respectively. MAb W6/32 recognizes a framework determinant of HLA class I antigens. B7/21.7 and S3/4 were kindly provided by Nancy Reinsmoen of the University of Minnesota, Minneapolis. OKIa1 and W6/32 were provided by John Sullivan of the University of Massachusetts Medical Center. A total of  $10^4$  <sup>51</sup>Cr-labeled target cells in 0.1 ml were incubated with 0.05 ml of 1:20 diluted MAbs for 30 min. The effector cells were then added in 0.05 ml and incubated for 6 h. The percent specific cell lysis was determined as described above.

## RESULTS

**HIV-1-specific cytotoxic activity in PBMC.** Fresh PBMC from an asymptomatic HIV-1-seropositive donor were tested for specific lysis of autologous B-LCL that were uninfected or infected with VAC, and the recombinant VAC/gag and VAC/gp160 as described in Materials and Methods. The K562 cell line was used to monitor natural killer cell activity (35). The results with different E/T ratios are presented in Fig. 1. No specific lytic activity of uninfected B-LCL and little lytic activity on VAC-infected targets were observed. Lysis of K562 cells was low. In contrast, we detected significant HIV-1 gag-specific and envelope-specific killer cell activity. No specific cytotoxic activities were detected with use of the PBMC of five HIV-1-seronegative normal individuals (data not shown).

**Establishment of HIV-1-specific CTL clones.** One month

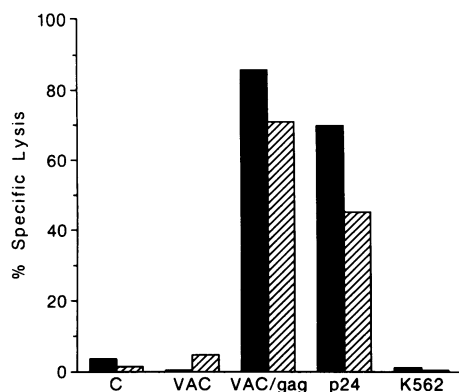


FIG. 2. Specificity of clone 165 for HIV-1 p24 protein. CTL clone 165 was tested for specific lysis of autologous B-LCL that were uninfected (C) or expressing wild-type VAC, recombinant *gag* (VAC/*gag*), or p24 protein. The K562 cell line was also used as another target cell. The CTL assay was performed in triplicate at E/T ratios of 10:1 (■) and 5:1 (▨).

after initiation of the culture, the developing clones were screened by testing for HIV-1-specific cytotoxic activity with autologous B-LCL that were uninfected or infected with VAC, VAC/*gag*, VAC/*pol*, and VAC/*gp160* and with the K562 cell line as a control. Two T-cell clones manifested specificity for HIV-1 envelope (data not shown), and the other clone (clone 165) was *gag* specific. There was no cytotoxic activity specific for the *pol* gene product, reverse transcriptase, and lysis of K562 cells was negligible. Phenotypic analysis showed that these cytotoxic clones were CD3+ CD8+ CD4- Leu11- (data not shown).

To further analyze antigenic specificity, we examined the ability of the *gag*-specific CTL clone 165 to lyse target cells pulsed with authentic HIV-1 p24<sup>gag</sup> protein purified from a recombinant *E. coli* strain. We observed that clone 165 significantly lysed B-LCL target cells pulsed with purified p24<sup>gag</sup> protein at levels comparable to the lysis of B-LCL infected with VAC/*gag*, but they did not lyse uninfected or VAC-infected targets (Fig. 2). We were unable to evaluate the two *env*-specific CTL clones from this donor for HLA restriction and epitope mapping because of their limited ability to proliferate in vitro.

**Characterization of an HIV-1 *gag*-specific CTL clone. (i) Determination of the CTL epitope in p24<sup>gag</sup>.** Fourteen overlapping synthetic peptides spanning the p24<sup>gag</sup> region were used to map the viral epitope recognized by CTL clone 165. As shown in Table 1, target cells pulsed with either synthetic peptide 1 or 2 were significantly lysed, denoting that the epitope recognized by this clone lies within the overlap region.

The fine specificity of CTL clone 165 was further examined by using truncated peptides corresponding to the first 20-amino-acid sequence of peptide 1 (Table 2, experiment A). Targets pulsed with peptides 90-17B and 90-17C lacking the five or seven residues at the N-terminal end of peptide 90-17 were observed to have identical high levels of lysis, whereas targets pulsed with peptides having carboxyl-terminal deletions of four residues from peptide 90-17 were lysed poorly (90-18, -18A, and -18B). Peptide 90-17A, which has the first 13 amino acids of this region deleted, was not recognized by the clone. Additional peptides containing amino acid truncations from the N and C termini revealed

TABLE 1. Lysis of target cells pulsed with synthetic peptides of HIV-1 p24<sup>gag</sup> by CD8+ CTL clone 165

Peptide		% Specific lysis <sup>a</sup>
No.	Residues <sup>b</sup>	
1	133-162	57.7
2	143-172	37.0
3	153-182	4.8
4-14	173-363	0

<sup>a</sup> Autologous B-LCL were pulsed with peptides at a final concentration of 50 µg/ml for 1 h before labeling with <sup>51</sup>Cr and used as targets for clone 165 in a standard cytotoxicity assay. Percent specific lysis was determined at an E/T ratio of 4:1.

<sup>b</sup> Residue numbering for the p24<sup>gag</sup> sequence is based on the HIV-1 (III B strain) sequence.

that the epitope for this *gag*-specific CTL is located within aa 145 to 150 (Table 2, experiment B).

(ii) **HLA restriction of cytotoxicity by CTL clone 165.** We first examined HLA restriction of the lysis of target cells infected with VAC/*gag* by CTL clone 165, using MAbs to HLA antigens. Anti-HLA class I MAb W6/32 inhibited the lysis of VAC/*gag*-infected target cells by CTL clone 165, and this inhibition was more apparent when a lower E/T ratio of 2.5:1 was used. The anti-class II MAbs to DP, DQ, and DR (B7/21.7, S3/4, and OKIa1, respectively) had no effect on the lysis of the target cells by this *gag*-specific CD8+ T-cell clone (Fig. 3). The results indicate that this CD8+ T-cell clone lysed target cells in an HLA class I-restricted manner.

The *gag*-specific clone 165 was also tested for its lytic activity on allogeneic B-LCL target cells infected with VAC/*gag* matched at a specific HLA class I locus or unmatched, as indicated in Fig. 4A. The HLA class I alleles of the donor of clone 165 are A3,25, B18, Bw62, and Cw3. This CD8+ clone was able to lyse two allogeneic target cells that shared Cw3 (Fig. 4A). Since target cells can also be sensitized to virus-specific lysis in vitro by incubation with the appropriate synthetic viral peptide (46), we examined the lysis of other allogeneic targets that had been pulsed with peptide 90-17C, the core peptide which contained the epitope recog-

TABLE 2. Fine specificity of the epitope recognized by CD8+ CTL clone 165

Peptide		Amino acid sequence	% Specific lysis <sup>a</sup>
No.	Residues <sup>b</sup>		
Expt A			
90-17	133–152	PIVQNIQGGMVHQAI SPRTL	67.7
90-17A	146–152	AISPRTL	−1.4
90-17B	138–152	IQGGMVHQAI SPRTL	65.0
90-17C	140–152	GGMVHQAI SPRTL	62.4
90-18	133–148	PIVQNIQGGMVHQAI S	9.6
90-18A	138–148	IQGGMVHQAI S	10.8
90-18B	140–148	GGMVHQAI S	3.1
Expt B			
LD-1	140–152	GGMVHQAI SPRTL	36.5
LD-3	145–151	QAISPRT	12.5
LD-4	145–150	QAISPR	25.2
90-17A	146–152	AISPRTL	−1.8

<sup>a</sup> Autologous B-LCL were pulsed with peptides at a final concentration of 50 µg/ml for 1 h before labeling with <sup>51</sup>Cr and used as targets for clone 165 in a standard cytotoxicity assay. Percent specific lysis was determined at an E/T ratio of 4:1.

<sup>b</sup> Residue numbering for the p24<sup>gag</sup> sequence is based on the HIV-1 (III B strain) sequence.

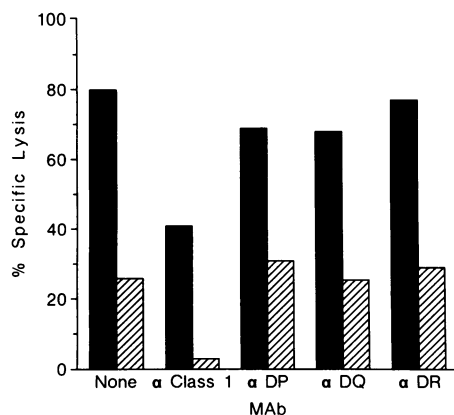


FIG. 3. Inhibition of HIV-1 *gag*-specific cytotoxicity by anti-class I MAb W6/32. Target cells ( $10^4$ ) were incubated with effector cells at E/T ratios of 10:1 (■) and 2.5:1 (▨) for 6 h in the presence of MAbs at a final dilution of 1:80. B7/21.7, S3/4, and OKIa1 were used as anti-HLA DP, anti-HLA DQ, and anti-HLA DR antibodies, respectively.

nized by this CTL clone. As shown in Fig. 4B, three of four peptide-sensitized target cells that share Cw3 were lysed. The failure to lyse one target cell that shared Cw3 may be due to variability in HLA alleles, because the HLA determinants which are recognized by the T-cell receptors differ from those defined by typing sera. This has been shown to be true for the HLA-A2 locus, where A2-restricted influenza A virus-specific CTL clones lyse some allogeneic target cells

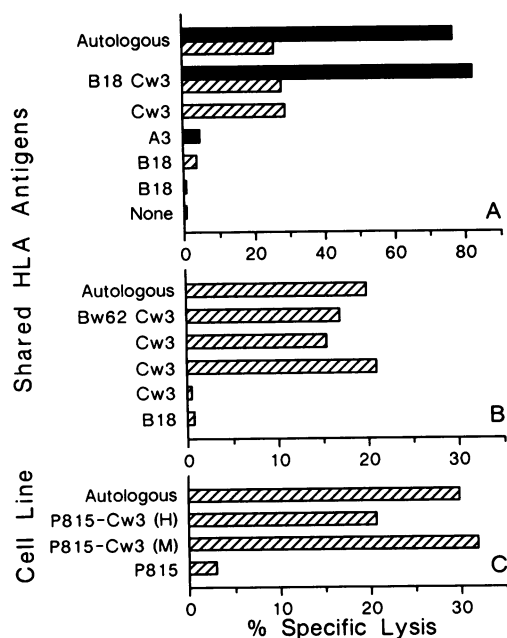


FIG. 4. MHC restriction of clone 165. Autologous and allogeneic targets matched at one or more HLA loci as indicated were infected with VAC/*gag* (A) or pulsed for 1 h with peptide 90-17C (B). HLA-Cw3-transfected P815 mouse mastocytoma cells and untransfected P815 cells pulsed with peptide 90-17C were also used as target cells (C). Percent specific lysis was determined at E/T ratios of 10:1 (■) and 3:1 (▨).

sharing an A2 locus but not other targets bearing A2 variants (2, 19). We were able to confirm the Cw3 restriction of clone 165 by using two transfected P815 (*H-2<sup>d</sup>*) mouse mastocytoma cells expressing human HLA-Cw3 that were pulsed with peptide 90-17C in a CTL assay. As shown in Fig. 4C, both cell lines expressing HLA-Cw3 were lysed by clone 165, while P815 control cells pulsed with the same peptide were not lysed. These data clearly show that the epitope on p24<sup>gag</sup> identified by clone 165 is definitely restricted by Cw3.

## DISCUSSION

We described a CD8<sup>+</sup> cytotoxic T-cell clone, 165, which recognized an epitope that maps to a 6-amino-acid region (aa 145 to 150) of HIV-1 p24<sup>gag</sup>. This epitope is located within a region of highly conserved amino acid sequence among various HIV-1 isolates, with most strains (BH10, HXB2, BH5, PV22, HAN, NDK, Z2, BRU, JH3, CDC4, ELI, WMJ, SF2, RF, MN, MAL, and CDC-451) having complete homology and only one isolate (OYI) having an alanine-to-proline substitution at position 146 (24). Interestingly, this HLA-Cw3-restricted CD8<sup>+</sup> CTL epitope overlaps B-cell epitopes of p24<sup>gag</sup> (aa 135 to 149 and 145 to 159) (23). However, this site would not have been predicted by Berzofsky's amphipathicity algorithm or by any of the widely used computer models (7, 37, 43). This fact underlines the importance of definitively identifying CTL epitopes by using T-cell clones.

CTL responses to HIV-1 *gag* protein mediated by CD8<sup>+</sup> positive HLA class I-restricted T cells have previously been reported (5, 17, 26, 36, 48). In some studies they were detected in variable degrees in fresh PBMC from HIV-1-infected individuals without in vitro stimulation. Walker et al. (48) reported low *gag*-specific CTL in three of eight HIV-1-seropositive patients, while Riviere et al. (36) and Koup et al. (17) found *gag*-specific CTL activity in over 80% of tested HIV-1-seropositive subjects. In the study of Nixon et al. (26), *gag*-specific CTL activity was detectable only after in vitro mitogenic stimulation of the cells from HIV-infected individuals.

Despite these bulk culture CTL responses, only a few epitopes have been identified on HIV-1 *gag*. With use of computer program-predicted *gag* epitopes, HLA-A2-restricted epitopes were reported by Claverie et al. (5) in bulk culture experiments using four sites within *gag* different from that detected by clone 165, one of which was conserved. Nixon et al. (26) used phytohemagglutinin-stimulated PBMC of a seropositive donor that had been cultured for 7 days and tested for lytic activity against target cells pulsed with synthetic HIV-1 *gag* peptides. A CTL epitope restricted by HLA-B27 was defined that comprises aa 265 to 279 and is highly conserved among HIV-1 isolates.

In contrast, sequence variation in the HIV-1 *env* protein raises concerns associated with their potential use in subunit vaccines based on *env* alone. For instance, an HLA-DR-restricted gp120-specific clone directed against an epitope spanning residues 410 to 429 of gp120 demonstrated very limited cross-reactivity against a range of HIV-1 variant peptides (40). Thus, our results with the p24-specific T-cell clone 165 and those of Nixon et al. (26) suggest that *gag* sequences that contain highly conserved T-cell epitopes may be very valuable and useful for inclusion in a combination vaccine for HIV.

The isolation of an HIV-1 *gag*-specific CD8<sup>+</sup> T-cell clone from an HIV-1-seropositive and asymptomatic donor is consistent with the *gag*-specific CTL activity that was ob-

served in the freshly isolated PBMC. This indicates ongoing antigenic stimulation *in vivo* since this cytotoxic activity was detected directly in fresh blood and continued to be detected in the cultured lymphocytes without *in vitro* HIV-1 antigenic stimulation. This T-cell clone has demonstrated a persistent *gag*-specific cytotoxic activity for the last 14 months and is presumably not polyclonal since it recognizes a single small peptide.

The highly conserved epitope on HIV-1 p24 is recognized by the CD8<sup>+</sup> clone 165 in an HLA-Cw3-restricted manner. It has been assumed that HLA-C major histocompatible antigens function similarly to the structurally related HLA-A and -B antigens (13). This is the first report to our knowledge that HLA-C antigens are restrictive elements for human CTL, although it has been demonstrated recently that transgenic mice expressing the HLA-Cw3 gene developed CTL responses to influenza and Sendai viruses which were Cw3 restricted (9). Cw3 is found in 20% of Caucasians and blacks, 62% of Native Americans, and 47% of Asians, and thus it is a common HLA allele (45).

In general, CTLs can eliminate virus-infected cells, as has been proven by the recovery from acute infections with viruses such as influenza virus (20–22), lymphocytic choriomeningitis virus (3), cytomegalovirus (33, 35), varicella-zoster virus (41, 42), and herpes simplex virus (25, 27) and in persistent (lymphocytic choriomeningitis virus) infections (28). Whether the role played by these CTLs is deleterious (18, 32, 38, 39) or helpful to the HIV-infected host is unclear. Their beneficial role is suggested by studies showing that the level of CD8<sup>+</sup> CTL detected in the blood of antibody-positive HIV-1-infected individuals appears to decrease with disease progression (30, 31), and this parallels the loss of HIV-1-specific cytotoxicity (47). Others have shown that CD8<sup>+</sup> lymphocytes isolated from HIV-infected individuals and simian immunodeficiency virus-infected macaques suppress HIV replication *in vitro*, although the mechanism of antiviral activity remains to be defined (15, 51). It has also been shown that synthetic viral lipopeptides can efficiently prime animals for MHC class I-restricted CTL responses (8).

While the biologic relevance of human CTLs in HIV-1 infections is not certain, data in animal systems indicate that they are likely to be important. Thus, there is a necessity to collect a wide data base of highly conserved epitopes. If it is shown that CTL responses to conserved epitopes provide protection in primate models against disease, it may be useful to combine several cross-reactive defined epitopes that may induce protection in the majority of individuals against HIV-1 disease.

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